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Preliminary Notes on the Culturing of *Gymnodinium brevis* Davis

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The mass mortality of fish off the west coast of southern Florida has attracted widespread attention since 1946. These catastrophes, commonly called Red Tides, have been associated with the high incidence of the marine dinoflagellate *Gymnodinium brevis* Davis (*1*) in the plankton populations (*2, 3*). Other investigators have reported conditions associated with these plankton blooms (*4–8*), but the study of this protist has been curtailed because of its sporadic occurrence in nature and the difficulty of maintaining it in laboratory culture.

Preliminary field investigations and culturing of a related species, *Gymnodinium splendens,* provided information that simplified the culturing of *G. brevis.* A medium composed of sea water, vitamin B_{12} (0.05 μg/100 ml), and Van Niel's (*9*) medium for sulfur bacteria (diluted 500 times) produced a culture containing more than 2500 cells of *G. splendens* per milliliter from an initial inoculum of two cells per milliliter in about 6 wk. *Gymnodinium brevis* responded better when the components of Van Niel's sulfur bacteria medium were diluted 1000 times and the vitamin B_{12} was increased to 0.1 μg/100 ml. The vitamin-B_{12} requirement of algal flagellates is discussed by Hutner and Provasoli (*10*). Vitamin B_{12} was required by dinoflagellates grown in pure culture by Provasoli *et al.* (*11*) and by Sweeney (*12*).

Using the latter medium as a base and a standard, we tested inorganic compounds, organic compounds, vitamins, and extracts of materials known to occur in nature, for example, soil and fish. By adding Florida peat extract, results were improved, but repeated experiments gave inconsistent results. A hot-water extract of beef liver further increased growth. We later substituted thiamine and biotin for the liver extract. The addition of the disodium salt of ethylenediamine tetraacetic acid (EDTA · Na), a metal buffer, (also designated ethylenedinitrilo tetraacetic acid disodium salt) increased the reliability of growth and resulted in the medium of Table 1. Soil extract, a more generally available material, can be substituted for Florida peat extract with a slight reduction in growth.

The conditions that we employed for culturing *G. brevis* are as follows: (i) Screw-cap test tubes 15 mm by 120 mm without liners were used for most experiments. Mass cultures are grown in erlenmeyer flasks and 5-gal Pyrex bottles with polyethylene covers (prepared by heating polyethylene sheet until soft and then molding over the mouth of the container). The glassware is cleaned by immersion in alcohol and sodium hydroxide solution, allowed to simmer 30 to 40 min in a solution of Cleaning Compound, No. 3298 (Arthur H. Thomas Co.), and rinsed 10 times with tap water and 15 times with distilled water. Extensive rinsing is employed since erratic results may be caused by minor contamination. (ii) Sterilization by steaming for 30 min on three successive days is optimum for the growth of *G. brevis.* McLaughlin (*13*) shortens the time by allowing 4-hr intervals between three 1-hr steaming periods without affecting growth results. Autoclaving produces erratic results. Growth is more consistent if inoculation is delayed for 4 days after sterilization. (iii) Aged sea water from the Gulf of Mexico [salinity, 36.5 parts per thousand (ppt)] was used in all experiments. The salinity of natural water containing high concentrations of *G. brevis* is usually between 32 and 34 ppt. A medium prepared according to Table 1 has a salinity of approximately 32 ppt. (iv) The temperature was usually maintained between 26° and 28°C, with as little variation as possible. (v) The *p*H stabilizes between 7.7 and 7.9 after sterilization. The *p*H of our aged sea water was between 8.1 and 8.2. The decreased *p*H is brought about by adding EDTA · Na. (vi) White daylight fluorescent tubes, 40-w, were used to maintain a light intensity of 175 to 300 ft-ca. (vii) Neither agitation nor aeration is required for growth. (viii) *G. brevis* was isolated into a unialgal culture in September 1953 and has been continuously subcultured since that time. Cultures usually reach maximum growth in 6 wk.

By substituting a group of trace metals (Fe, Zn, Mn, Sr, Rb, Mo, Co, Cu, Cr, Ti, Al, Si, and Zr) for soil extract, we obtained a medium that supports good initial growth of *G. brevis,* but continuous subculturing has not been successful. Titanium and zirconium were added to the mixture, although they are not normally present in measurable amounts in sea water (*14*), because these metals were found abundantly in water of *G. brevis* blooms (*7*).

Investigations led us to believe that heavy rainfall and subsequent river discharge are instrumental in

Table 1. Medium for the unialgal isolation of *G. brevis* Davis. After all additions are made, there is approximately 110 ml of medium.

Aged sea water (salinity, about 36.5 ppt)	95.0 ml
Distilled water*	5.0 ml
NH_4Cl†	0.1 mg
KH_2PO_4†	0.05 mg
$MgCl_2 \cdot 6H_2O$†	0.02 mg
$NaHCO_3$†	0.1 mg
$Na_2S \cdot 9H_2O$†	0.1 mg
Vitamin B_{12}	0.1 μg
Thiamine hydrochloride	1.0 mg
Biotin	0.05 μg
Soil extract‡	2.0 ml
EDTA · Na (6 ml of 0.25-percent solution)	15.0 mg

* More or less may be required, depending on the salinity.
† Added as 0.5 ml of the following solution of the components of Van Niel's medium for sulfur bacteria: NH_4Cl, 0.2 g; KH_2PO_4, 0.1 g; $MgCl_2 \cdot 6H_2O$, 0.04 g; $NaHCO_3$, 0.1 g; and $Na_2S \cdot 9H_2O$, 0.2 g to 1 lit of distilled water.
‡ Simmer for 40 min a mixture of 500 g of garden soil and 1 lit of distilled water. Let it stand for 4 days and decant the supernatant. Repeat simmering and decantation until extract is clear. Our soil gives a yellowish-brown extract.

initiating a bloom of *G. brevis* (*8, 15, 16*). On the basis of this belief, we conducted culture experiments whereby mediums were prepared by adding river water, extracts of peat soils from the western Florida coastal region, or both, to sea water from the Gulf of Mexico. Sea water containing 4 to 10 ml of river water per 100 ml supported limited growth of *G. brevis*. Seven to 10 ml of river water or peat extract per 100 ml of sea water is better, and peat extract or mixtures of peat extract and river water at this concentration are more productive than river water alone. The addition of vitamin B_{12} (0.05 μg/100 ml of medium) improved the reproducibility and growth.

The conditions in a bloom of *G. brevis* often led us to believe that fish which had been killed and subsequently decayed imparted nutrients that helped to perpetuate the bloom. We designed culture experiments to determine whether extracts of partially decayed fish promoted growth. The results of these experiments indicate that the extracts contain one or more growth-promoting substances. Robinson (*17*) lists fish, or portions thereof, as containing vitamins B_{12}, biotin, thiamine, and other B-complex vitamins.

Laboratory cultures of *G. brevis* have attained homogeneous concentrations exceeding 2 million cells per liter. This concentration is far below the highest report (*2, 3*); however, the values cannot be compared. Among other reasons, *G. brevis* in cultures concentrate to form masses that we disperse by shaking before making counts. A similar tendency to concentrate, but on a larger scale, may be expected in nature. Counts of a sample from such a concentration would be high. Ketchum (*5*) considered a tendency of the organism to concentrate as a possible cause of high surface organic phosphorus concentrations.

Mass cultures of *G. brevis* maintained in this laboratory are toxic to fish. We conducted toxicity experiments using *Mollienesia sp.* and *Mambras vagrans* as test fish. Controls consisted of water taken at the collecting point of the fish and unialgal cultures of *G. splendens* containing more than 2.5 million cells per liter. All fish died in the water containing *G. brevis*, but none died in either of the controls. This experiment was repeated with the same results (*18*).

References and Notes

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